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Methoxychlor and Estradiol-17β Affect Alevin Rainbow Trout (*Oncorhynchus mykiss*) Mortality, Growth, and Pigmentation

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Since the pesticide methoxychlor [MXC;1,1, 1-trichloro-2,2-bis(*p*-methoxyphenyl) ethane] has been shown to have low toxicity in mammals, it is used as a substitute for DDT to control insect pests. The half-life of MXC in soil, however, is greater than 6 months (Anonymous 1988) and it bioaccumulates in the environment suggesting that it may pose a threat to non-target organisms (Plumb 1993).

Various studies have shown that MXC may elicit estrogenic responses in mammals. Duax and Weeks (1980) have shown that the estrogenic activity of MXC is due to its metabolism by liver microsomes to HPTE [2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane] which competes for the estrogen receptor. Exogenous estradiol-17β (E_2) administration has been shown to affect salmonids by decreasing juvenile rainbow trout (*Oncorhynchus mykiss*) growth and survival (Johnstone et al. 1978; Krisfalusi and Cloud 1996) and by disrupting gonadotropin release from the pituitary gland (Benfey et al. 1989).

The 96 hr LC_{50} for MXC ranges from 5-40 µg/L for rainbow trout suggesting that fish are susceptible to this pesticide (Heming et al. 1989). Investigators have reported that MXC treatment of immature flagfish (*Jordanella floridae*) (Holdway and Dixon 1986), larval white sucker (*Catostomus commersoni*) (Holdway et al. 1987), and juvenile rainbow trout (Krisfalusi et al. 1998) decreased growth and increased mortality. Preliminary observations (unpublished data) have indicated that MXC administration lightens the color of alevin rainbow trout skin. Juveniles may be more sensitive to environmental contaminants compared to adults because this is a period of rapid growth and development, and because of the limited development of organs involved with detoxification (Laale 1981; Holdway and Dixon 1986). Because the rate of conversion of MXC to HPTE in embryonic salmonids is unknown, and since the responses elicited by MXC have never been directly compared to the responses elicited by exogenous E_2, the objective of this investigation was to determine whether MXC and E_2 elicit similar responses by examining the acute toxicities of these chemicals and their effect on alevin rainbow trout growth and development. The hypothesis to be tested was that MXC and E_2 would effect rainbow trout in a similar fashion.

MATERIALS AND METHODS

Rainbow trout gametes were obtained from Mt. Lassen Trout Farms (Red Bluff, CA) and eggs were fertilized by methods previously described (Nilsson and Cloud 1992). Embryos were incubated in PVC boxes equipped with mesh bottoms in a Heath incubator (Heath Techna, Kent, WA) at 11-12° C. To examine the effects of

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MXC or E_2 on growth, survival and pigmentation, starting 6 days prior to hatching (about 25 days after fertilization), embryos were partitioned into 10 treatment groups; each treatment group had 3 replicates of 50 fish. Embryos were immersed in aerated aged tap water (untreated), carrier-control water containing the highest concentration of dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO) used as a vehicle (0.5 mL/L DMSO), 0.5, 1, 2, or 4 mg/L 95% pure MXC (Sigma), or 0.5, 1, 2, or 4 mg/L E_2 (Sigma). The commercially available pesticide contains various contaminants, thus purified MXC was used to examine the effects of the single compound. Embryos were treated for 2 hr periods every third day for a total of 10 treatments (day 0 represents hatching). Following immersion, fish were rinsed to remove excess chemical, and checked daily for mortality. To explore pigmentation differences, at 15 days post-hatching (corresponding to the 8th treatment with MXC or E_2), 10 live fish from each group were photographed and 5 fish from each replicate were sacrificed by overdosing with tricaine methanesulfanate (MS-222). Alevins were fixed in 10% neutral buffered formalin and lateral mid-body skin samples were observed for differences in melanophore number and morphology via light microscopy. The experiment was terminated 24 days post-hatching (this period of development corresponds to the tune of first feeding) and surviving fish were sacrificed, wet weights recorded, and final mortality rates calculated.

Because the mortality of E_2-treated fish was much greater than MXC-treated fish, an additional experiment was completed to explore this response. Rainbow trout were exposed to either 10 mg/L MXC or 0.5 mg/L E_2 for 2 hr periods, every-other-day for 15 treatments. The concentrations of E_2 and MXC used for this experiment have been previously shown to cause approximately equal mortality (unpublished data). The experimental design is shown in Table 1.

Table I: Experimental design for rainbow trout exposed to either 10 mg/L MXC or 0.5 mg/L E_2. Each group began with 3 replicates of 50 embryos. Treatment groups included control (no exposure), 6 groups exposed to MXC, and 6 groups exposed to E_2. Treatment began 4 days prior to hatching and continued through 24 days after hatching.

	Developmental Time (days after hatch)														
Group	**-4**	**-2**	**0[a]**	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**	**22**	**24**
Control															
MXC #1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
MXC #2			*	*	*	*	*	*	*	*	*	*	*	*	*
MXC #3					*	*	*	*	*	*	*	*	*	*	*
MXC #4							*	*	*	*	*	*	*	*	*
MXC #5									*	*	*	*	*	*	*
MXC #6											*	*	*	*	*
E_2#1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E_2#2			*	*	*	*	*	*	*	*	*	*	*	*	*
E_2#3					*	*	*	*	*	*	*	*	*	*	*
E_2#4							*	*	*	*	*	*	*	*	*
E_2#5									*	*	*	*	*	*	*
E_2#6											*	*	*	*	*

* Indicates exposure to chemical

[a] Day 0 indicates hatching

Data are expressed as the mean ± SD. Statistical analyses were based on a one-way ANOVA followed by Duncan's new multiple range test ($p<0.05$). ANOVA

showed no significant differences between replicates for weights; thus, replicate measurements for individual treatment groups were pooled and total means utilized for analysis. Where appropriate, percent mortality data were transformed (arcsin $\sqrt{\pi}$; where π represents percent mortality) for statistical analysis (prior to ANOVA as the assumption of equal population variances could not be accepted) and transformed back for graphical representation. Linear regression was completed to determine if chemical concentration was correlated with weight or mortality.

RESULTS AND DISCUSSION

Immersion in MXC (r= 0.91) or E_2 (r= 0.84) increased rainbow trout mortality in a dose-dependent manner; however, E_2-treated fish had significantly higher mortality compared to MXC-treated fish (Fig. 1). Immersion in E_2 resulted in mortality rates more than 3 times greater than MXC-treated fish exposed to similar chemical concentrations. Figures 2a and 2b indicate when, during the exposure regimen, the mortality occurred for MXC and E_2-treated rainbow trout respectively. Methoxychlor treated fish showed a drastic increase in mortality at 16 days post-hatching despite the number of exposures preceding this developmental period. Conversely, E_2-treated fish exhibited an increase in mortality following 10 exposures irrespective of developmental time.

In this study, treatment with increasing concentrations of MXC resulted in a dose-dependent increase in mortality, yet there was not a significant difference in the survival rates of untreated and MXC-treated fish. This is consistent with findings from Heming et al. (1988), in which it was reported that a single 2 hr treatment ($\leq$ 580 µg/L) with commercial MXC (Sanex MXC 25E) had no effect on the survival of alevin rainbow trout. The lack of difference in mortality found in the present investigation, however, is attributed to the elevated mortality reported for the untreated group. This mortality occurred very early in the experiment whereas the mortality due to chemical treatment manifested only after several exposures. Since the DMSO-treated fish did not show increased mortality early in the experiment, and since DMSO was used as the vehicle for chemical treatment, DMSO-treated groups may be more appropriate to use for comparison. The present investigation showed mortality of fish treated with the 3 highest doses of MXC was significantly higher than DMSO-treated fish. These data are consistent with results indicating that MXC increases mortality in larval white sucker, immature flagfish (Holdway and Dixon 1986; Holdway et al. 1987), and juvenile rainbow trout (Krisfalusi et al. 1998). The high mortality observed in E_2-treated fish is consistent with previous findings by Herman and Kincaid (1988); however, the dose-dependent response to E_2 has not been previously reported in alevin rainbow trout.

Since it has been reported that several fish are intolerant to MXC, the 96 hr LC_{50} for various teleosts ranges from 10-260 µg/L (Heming et al. 1989), the relatively low mortality in this study is an enigma In mammalian systems, the toxicity reported for MXC appears to be due to HPTE, a metabolite of this pesticide, and not due to MXC directly (Duax and Weeks 1980). Additionally, many studies documenting the toxicity of MXC have been completed using the commercial pesticide. The decreased mortality noted in our investigation may have resulted because pure MXC is not as toxic to rainbow trout as the commercial pesticide and/or because alevin rainbow trout lack the enzymes required to metabolize MXC into more active/toxic metabolites.

Figure 1 depicts the difference in survival between MXC and E_2-treated fish. Additionally, it should be noted that the highest mortality occurred following the 8th

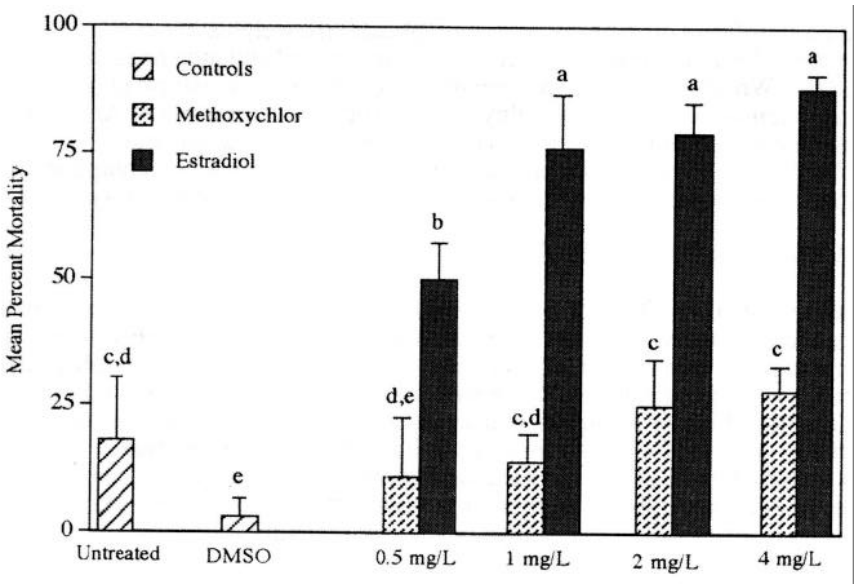
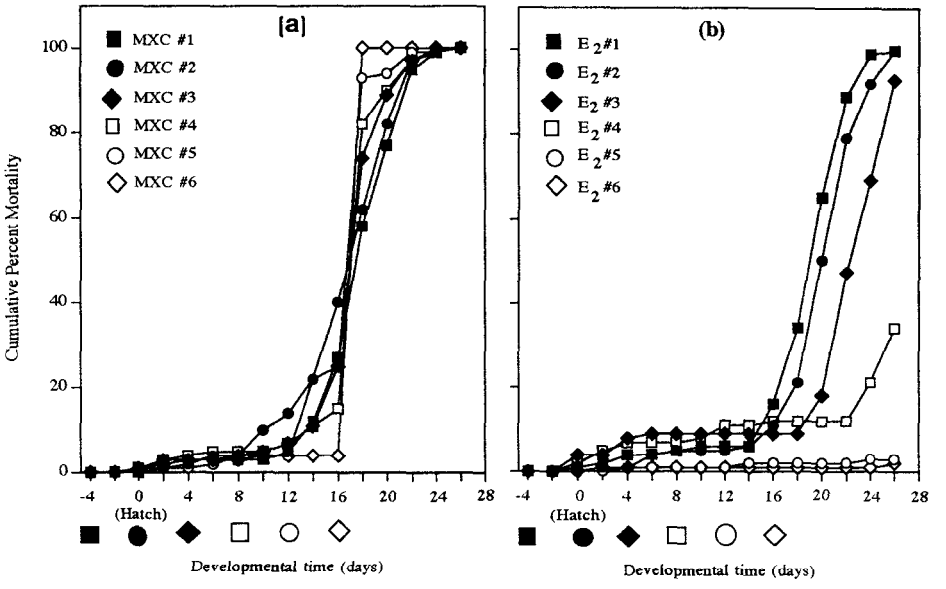


Figure 1. Mean percent mortalities for rainbow trout 24 days post-hatching. Rainbow trout were immersed in chemical every third day for a total of 10 exposures. Each treatment group consisted of three replicates of approximately 50 fish. Data represent means ± SD. Different letters indicate that means are significantly different at $p < 0.05$.

chemical exposure (16 days post-hatching) (data not shown). It was not clear why this massive mortality occurred, which resulted in nearly complete eradication of those groups treated with the 3 highest concentrations of E_2, but two explanations seemed plausible. The increased mortality may have resulted from chemical bioaccumulation that reached a threshold concentration around the 8th treatment. Alternatively, the mortality may have been extreme following the 8th chemical exposure as this may be a period of development in which rainbow trout embryos are extremely sensitive to external stressors.

To explore these two possibilities, rainbow trout were treated with either 10 mg/L MXC or 0.5 mg/L E_2 as described in the methods section. Those fish immersed in MXC showed a pattern of mortality consistent with the idea that there is a period of development during which rainbow trout are extremely sensitive to MXC (Fig 2a). Despite the number of immersions (ranging from 1 to 11), at 16 days post-hatching a drastic increase in mortality was observed in nearly all 6 groups. In addition, those groups exposed to MXC the greatest number of times appeared to acquire a tolerance to this compound. For example, at 16 days post-hatching MXC group #l (immersed 11 times) had the smallest increase in mortality while MXC group #6 (immersed once) showed the largest increase. These findings are similar to previous observations (Holdway et al. 1987) in which white sucker eggs exposed to MXC increased MXC tolerance of resulting larvae. However, exposure of flagfish eggs to MXC decreased the tolerance of juveniles to this pesticide (Holdway and Dixon 1986). The pattern of mortality observed for E_2-treated rainbow trout was consistent with the hypothesis of bioaccumulation (Fig. 2b). Estradiol reached a threshold concentration between the 10th and 11th exposures;



Figures 1(a) and (b). Mean cumulative percent mortalities for rainbow trout treated with 10 mg/L MXC (a) and 0.5 mgL E_2(b). Symbols beneath specific developmental days indicate when a particular group started the exposure regimen. Once treatment for an individual group was initiated, exposures were every-other day until 24 days post-hatch. Each point represents the mean mortality for the three replicates within each group.

alevins showed a predictable increase in mortality following the 10th exposure independent of developmental progress. Although MXC or E_2 both increased rainbow trout mortality, these chemicals appeared to act via different mechanisms.

At 24 days post-hatching groups treated with MXC or E_2 weighed less than untreated and DMSO-treated groups (Fig. 3). In MXC-treated groups there was **a** negative correlation between concentration and wet weight (r= -0.83) while in the E_2-treated groups there was not. Fish treated with MXC had mean weights ranging from 58 to 79 mg; mean weights of E_2-treated fish ranged from 68 to 71 mg. The decrease in growth is consistent with previous studies that have shown exogenous E_2 administration reduces rainbow trout growth (Johnstone et al. 1978; Herman and Kincaid 1988; Krisfalusi and Cloud 1996) and that MXC exposure decreases larval white sucker, immature flagfish (Holdway and Dixon 1986; Holdway et al. 1987), and rainbow trout (Krisfalusi et al. 1998) growth. Weights were recorded prior to initiation of feeding, thus all were equal relative to available nutrients. Therefore, the stunted growth of chemically-treated fish may have resulted from a decreased efficiency by which yolk products were metabolised into usable resources.

Although the data indicate that E_2-treated fish showed no correlation between steroid concentration and wet weight, the massive mortality in groups treated with the 3 highest doses of E_2 may mask such a response. The average weight of those embryos treated with the 4 concentrations of E_2 was the same, but was significantly greater than those embryos treated with 2 or 4 mg/L MXC. Since 50 to 75% of the

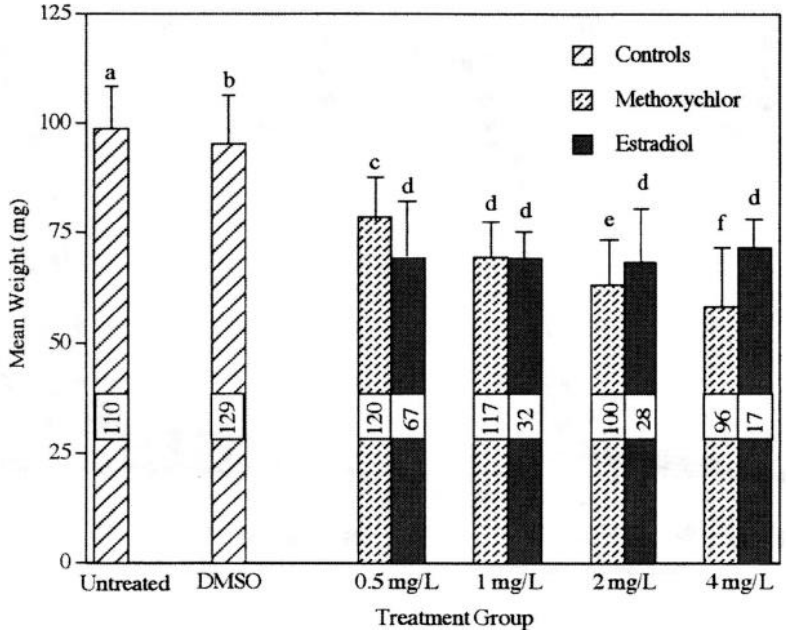


Figure. 3. Mean wet weights of rainbow trout sacrificed 24 days post-hatching. Data represent means ± SD. The number within each bar represents the sample size for that group. Different letters indicate that means are significantly different at $p < 0.05$.

embryos in the E_2-treated groups failed to survive, one interpretation of the data is that E_2 selectively killed smaller fish and those that survived E_2 treatment were larger. This may account for the relatively uniform size of E_2-treated fish.

By 6 days post-hatching (immersion 5) the skin of MXC-treated fish was lighter in color compared to untreated fish. Conversely, the E_2-treated fish appeared darker in color compared to untreated fish, but this difference was noted between 9-12 days post-hatching (between immersion 6 and 7) (Fig. 4a-c). Though several exogenous factors affect pigmentation in fish, the neuroendocrine system also regulates this event. Since the skin of MXC-treated fish was lighter, and the skin of E_2-treated fish was darker in color compared to untreated groups, it seems that these two compounds may have different mechanisms of action. The lighter skin of MXC-treated fish may be due to decreased synthesis/release of melanophore stimulating hormone (MSH) from the pituitary gland. Because MSH is involved in pigment dispersion in ectotherms, disruption of this hormone could produce lighter skin in the MXC-treated fish. Conversely, the darker skin of E_2-treated fish may indicate increased toxicity; several species of fish exhibit a darker skin color when in poor health (Plumb 1993). The difference in pigmentation observed in the MXC or E_2-treated fish was only temporary as all fish had similar pigmentation by 21 days post-hatching (data not shown) indicating that these compounds are modifying, but not permanently changing the neuroendocrine system. By counting melanophores in randomly selected regions of skin, it was concluded that untreated, MXC and E_2-treated rainbow trout had the same number of melanophores per area of skin; however, the melanin within the melanophores of the untreated and E_2-treated fish appeared to be more dispersed when compared to MXC-treated fish (Fig. 4d-f).

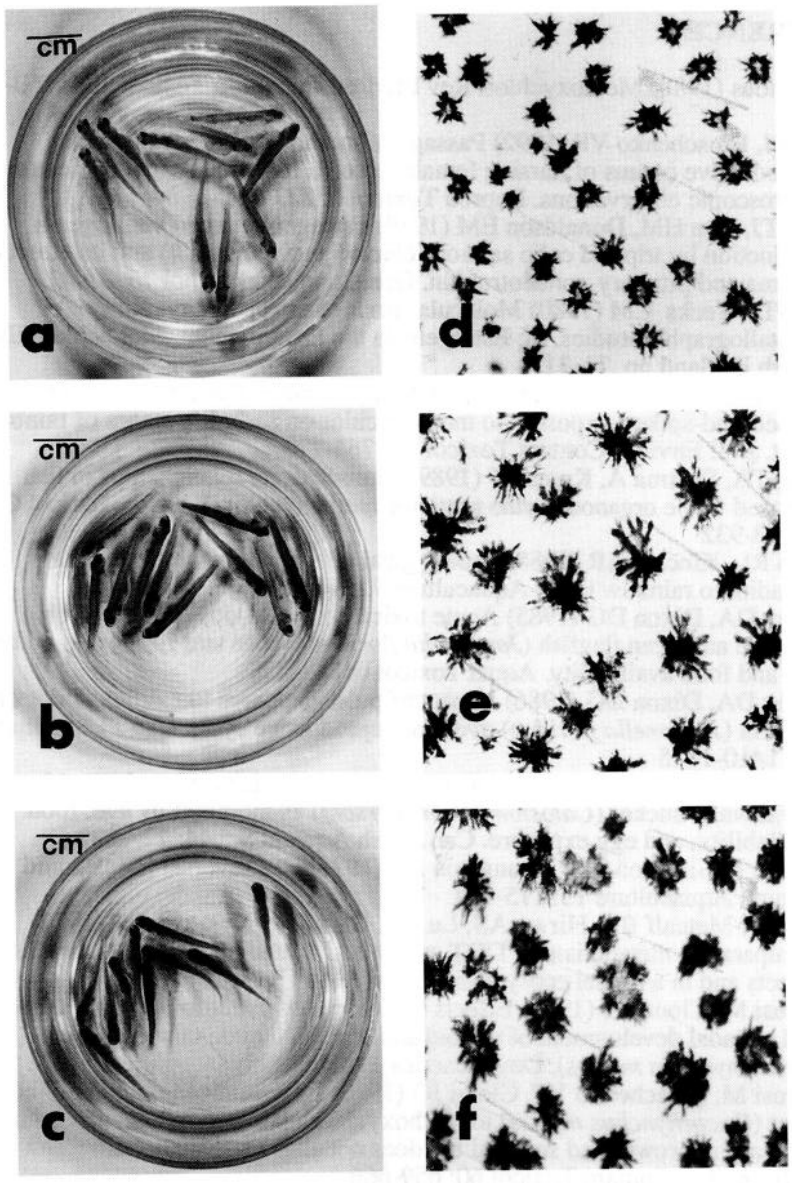


Figure 4. Rainbow trout photographed 15 days post-hatching. Whole rainbow trout exposed to 2 mg/L MXC (a), untreated (b), and 2 mg/L E_2 (c). Each group had a sample size of ten fish. (d), (e), and (f) are photographs of whole mount skin samples from fish treated with 2 mg/L MXC, untreated, and 2 mg/L E_2 respectively viewed at a magnification of 130x. Fish exposed to all four concentrations of MXC and E_2 looked similar, thus the 2 mg/L groups are representative samples.

In summary, this investigation showed that MXC and E_2 affected rainbow trout survival, growth, and skin pigmentation; however, since the effects generated by MXC do not mimic the effects caused by E_2, it is suggested that these two compounds may act via different mechanisms. Further investigation into the mechanism(s) of MXC action is underway.

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